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Use of PROAST software to assess the influence of decabrominated diphenyl ether and/or cadmium on thyroid hormones homeostasis in rats

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Abstract

In relation to commonly used no-observed-adverse-effect-level (NOAEL) point of depature, because of its statistical power and reliability, Benchmark dose (BMD) approach has been proposed as an alternative in dose-response assessment. The aim of this study was to derive BMD (10%) by means of PROAST software to quantify influence of cadmium (Cd) and/or decabrominated diphenyl ether (BDE209) on thyroid hormones homestasis. Study was conducted on male *Wistar* rats treated orally by gavage for 28 days by either single substances or their combination. Three groups of

animals were dosed with Cd at levels of 2.5, 7.5 and 15 mg/kg b.w./day, three groups of animals were dosed with BDE209 at levels of 1000, 2000 or 4000 mg/kg b.w./day, while 9 groups received different dose mixtures of previously given dose levels of Cd and BDE209 (design 3 x 3). Results of the study have indicated that Cd+BDE209 mixtures are likely more potent to disrupt thyroid function than would be expected from the chemicals individually. Derived BMD – lower confidence limits (BMDL), if ratio BMD/BMDL is < 10, were 9.4 mg Cd/kg b.w./day and 2155 mg BDE209/kg b.w./day for the effect on T3; and 6.22 mg Cd/kg b.w./day in the mixture with BDE209 2000 mg/kg b.w./day for the effect on FT3.

Key words: dose-response; decabrominated diphenyl ether; cadmium; thyroid hormones; mixture.

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Introduction

Humans and all other organisms are typically exposed to multi-component chemical mixtures, present in the surrounding environmental media (water, air, soil), in food or in consumer products. However, chemical risk assessment usually considers the effects of single substances in isolation, an approach that is only justified if the exposure to mixtures does not bear the risk of an increased toxicity. Furthermore, there is strong evidence that chemicals with common specific modes of action work together to produce combination effects that are larger than the effects of each mixture component applied singly (1-3). Fewer studies have been conducted with mixtures composed of chemicals with diverse modes of action (4, 5), with results clearly pointing in the same direction: the effects of such mixtures are also higher than those of the individual components. Of particular interest, as reported within the findings of a project on mixture toxicology and ecotoxicology commissioned by the European Commission, DG Environment (4, 5) is to examine joint action chemicals of which cadmium (Cd) as relatively known environmental contaminant and novel one decabrominated diphenyl ether (BDE209) are the subject of the present work. It has been demonstrated that acute or chronic Cd

exposure could result in toxic effects especially associated with targets such as liver, kidney, bone, lungs and testes in humans and experimental animals (6, 7). The available literature provides some data concerning Cd effects on thyroid gland (8-11) as well as estrogen like effects, referred in recent publication of Imran et al. (2010) (12). Recent studies with BDE209 have shown that it disturbs hepatic enzyme activity and thyroid hormone function (13-17), causes a decrease in epididymal sperm functions (18), and has potential neurotoxic and neonatal risks (19, 20). However, there are no available data on Cd and BDE209 combined toxicity, which is of utmost interest and particularly in the light of thyroid disfunction as a subtle and critical event for neurodevelopmental toxicity of various chemicals (21).

Our recently published results based on mechanistic interpretation have indicated that Cd-BDE209 mixtures are likely more potent to disrupt thyroid function than would be expected from the chemicals individually (22). In order to exam dose-response relationship unlike tradionaly used no-observed-adverse-effect-level (NOAEL) approch, in this study we calculated benchmark dose for the effect of 10% towards controls (BMD10) recommended by European Food Safety Authority (EFSA) in 2009 (23) for analysing dose-response data from experimental studies. Namely, the EFSA Scientific Committee concluded that the BMD approach is a scientifically more advanced method to the NOAEL approach for deriving a Reference Point (RP). Further on, during last year EFSA released into public technical report on use of PROAST software packages for applying the BMD approach in risk assessment (24), which we applied to model the effect of BDE209 and Cd on T3, T4, FT3 and FT4 level in rat subacutely exposed to single chemical or their mixture.

2. Materials and methods

2.1. Experimental animals

Male albino *Wistar* rats weighing 200-240 g were obtained from the disease free stock of the animal house of Military Medical Academy, Belgrade. The animals were housed in plastic cages with plastic bottom and wire mesh top, in climate-controlled facility with a constant day–night cycle (12 h/12h light-dark ratio) at a temperature of 20-24 °C and relative humidity between 40 and 60%. Food and tap water were offered *ad libitum* throughout the study. The experimental animals were treated according to the Guidelines for Animal Study No. 9667-1/2011 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia).

After a quarantine period of 14 days, groups of 8 animals were treated orally by gavage for 28 days either by single substances or their combination in

a volume of 0.5 mL/kg bw. Three groups of animals were dosed with 2.5, 7.5 and 15 mg Cd/kg b.w./day, calculated to Cd (groups were assigned as Cd 2.5, Cd 7.5 and Cd 15, respectively), three groups of animals were dosed with 1000, 2000 or 4000 mg BDE209/kg b.w./day (groups were assigned as BDE209 1000, BDE209 2000 and BDE209 4000, respectively), while 9 groups received different dose mixtures of previously given dose levels of Cd and BDE209 (design 3 x 3). Cd salt and BDE209 were dissolved in dimethyl sulfoxide (DMSO) prior to application. Rats in the vehicle control group received by gavage the vehicle only (DMSO group), while animals in control group received certain amount of saline solution (control group). Doses of Cd used in experiment have been chosen to reflect environmental to occupational exposure (6, 25) doses of BDE209 have been chosen based on experiments where similar endpoints were examined (26).

Blood was collected after decapitation from the carotid arteries in glass tubes, and then centrifuged at 3000×*g* for 30 min. The supernatant (serum) was transferred to polypropylene test tubes and stored at −70 °C until thyroid hormones analysed.

2.2. Chemicals

Cadmium-chloride ($CdCl_2$ x H_2O) was purchased from Merck (Darmastadt, Germany) while BDE209 (98% pure) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Hormone analysis

Serum samples were analyzed for thyroid-stimulating hormone (TSH), thyroxin (T4), free thyroxin (FT4), triiodothyronin (T3) and free triiodothyronin (FT3) using commercial tests on Roche Elecsys 2010 (Roche Diagnostics, Mannheim, Germany). Values for T3 and T4 are expressed as nmol/L, for FT3 and FT4 as pmol/L while for TSH as mIU/L.

2.4. Statistical analysis

For the modelling of dose response relationship the data were analysed by the BMD approach, using the PROAST software (24, 27). In this approach, the dose–response data are statistically evaluated in general, by fitting a dose–response model to the data. In the present study associated BMD10 (10 % was used as the practical default) (23). For most parameters, a BMD at this level appears to approximate the limit between adversity and adaptivity, based on the pathophysiology of the effect under experimental conditions in the rat. In addition, this default level also seems to be close to the within-animal variation

(28, 29). The choice of the model for deriving the BMD follows from a procedure of selecting a model from the following nested family of models:

model 1: $y = a$; model 2: $y = a \exp(b\,x)$; model 3: $y = a \exp(b\,x\mathrm{d})$; model 4: $y = a\,(c-(c-1))\exp(b\,x)$ or model 5: $y = a\,(c-(c-1))\exp(b\,x\mathrm{d})$, assigned in figures as E1, E2, E3, E4 or E5, respectively. In these models the parameter *a* represents the background response; the parameter *b* reflects the 'slope' or the 'strength' of the response, *c* is maximal response relative to the background level, and d steepness (27). To account for statistical errors in the dose–response data, the confidence interval around the BMD (statistical lower confidence limit of BMD - BMDL) is calculated as well. The BMD/BMDL ratio was used as a measure for the (statistical) uncertainty in a data set. In case of a more than ten-fold difference between BMD and BMDL, the data were considered uninformative. For graphical presentation on a log-dose scale, an arbitrary value (but lower than the lowest dose) is used for plotting dose zero.

3. Results

In the present work dose-response relationship has been established and accordingly BMD10 and BMDL were derived for single chemical treatments as well as their mixture (Table I).

Exponential models were used for all data sets, while among them, model E4 was used in majority of cases. Exceptions were as follow: model E1 for the effect of BDE209 on FT3, and model E2 for the effects of Cd on T3, Cd+BDE209 2000 on FT3 and BDE209 on T3 and T4. Results obtained for TSH could not be used for further data analysis since all the values were below the limit of detection. Dose response model for the effect on T4 and FT4 did not change after adding different doses of BDE 209 to Cd, and for all tested sets of data model was the same, E4: $y = a\,(c-(c-1))\exp(b\,x)$. In the case of hormone T3 starting model E2 for Cd or E1 for BDE209, changed to the E4 for their mixture without further changes in a dose dependent manner. Unlike dose-response model for T3, effect of Cd on FT3, T4 and FT4 could be described by model E4. Concerning FT3 hormone, addition of BDE209 changed the model proposed for BDE209. However, in comparison with the model initially fitted for Cd, addition of the first dose of BDE209 in the mixture did not change the model, whereas the next two higher doses lead to model change. Concerning T4 and FT4, mixtures have been defined by E4 model. It should be mentioned that in case of T4 hormone proposed model for a single compound BDE209 has changed from E2 to E4 when combined with Cd. Illustration of model fitted for the effects of mixtures of Cd and BDE 209 on FT3.

Table I Benchmark dose levels for the influence of Cd and/or BDE209 on thyroid hormones homeostasis

Treatment		T3	FT3	T4	FT4
Cd	BMD	15.2	0.0454	0.5771	0.4929
	BMDL	9.4	0.0014	0.0030	0.0025
	BMD/BMDL	1.6	32.4	192.3	197.2
Cd + BDE209 1000	BMD	1.0	0.0462	0.0339	0.0323
	BMDL	0.0020	0.0012	0.0013	0.0013
	BMD/BMDL	500	38.3	26.1	24.8
Cd + BDE209 2000	BMD	0.9501	11	0.0290	0.0281
	BMDL	0.0018	6.2201	0.0017	0.0013
	BMD/BMDL	527.8	1.8	17.1	21.6
Cd + BDE209 4000	BMD	0.0972	0.0454	0.0339	0.0281
	BMDL	0.001	0.0013	0.0013	0.0013
	BMD/BMDL	97.2	34.9	26.1	21.6
BDE209	BMD	3266	/	16.7	18.9
	BMDL	2155	/	0.0270	0.0330
	BMD/BMDL	1.5	/	618.1	572.7

Cd - groups dosed with 2.5, 7.5 or 15 mg Cd/kg b.w./day;
Cd + BDE209 1000 - groups dosed with 2.5, 7.5 or 15 mg Cd/kg b.w./day and 1000 mg BDE209/ kg b.w./day;
Cd + BDE209 2000 - groups dosed with 2.5, 7.5 or 15 mg Cd/kg b.w./day and 2000 mg BDE209/ kg b.w./day;
Cd + BDE209 4000 - groups dosed with 2.5, 7.5 or 15 mg Cd/kg b.w./day and 4000 mg BDE209/ kg b.w./day;
BDE209 - groups dosed with 1000, 2000 or 4000 mg BDE209/kg b.w./day;
the BMD/BMDL ratio < 10 was considered to be of relevance (grey highlighted cells).

In general, BMDL decreased from Cd groups over Cd plus BDE209 groups (Table I). In the case of T3, decrease of BMDL is followed by an increase of BDE209 dose in the mixture and the lowest calculated BMDL for T3 (0.0010 mg Cd/kg b.w./day) was obtained for the mixture of Cd and the highest dose of BDE209. For FT3 the lowest BMDL was 0.0012 mg Cd/kg b.w./day, but for the mixture of Cd and the lowest dose of BDE209. The lowest

derived BMDL was 0.0013 mg Cd/kg b.w./day in the mixture with BDE209 1000 or 4000, for the effect on T4 hormone. For the all mixtures of chemicals, derived BMDL for the effect on FT4 was 0.0013 mg Cd/kg b.w./day.

Illustration of model fitted for the effects of mixtures of Cd and BDE209 on FT3 is presented on the Figure 1.

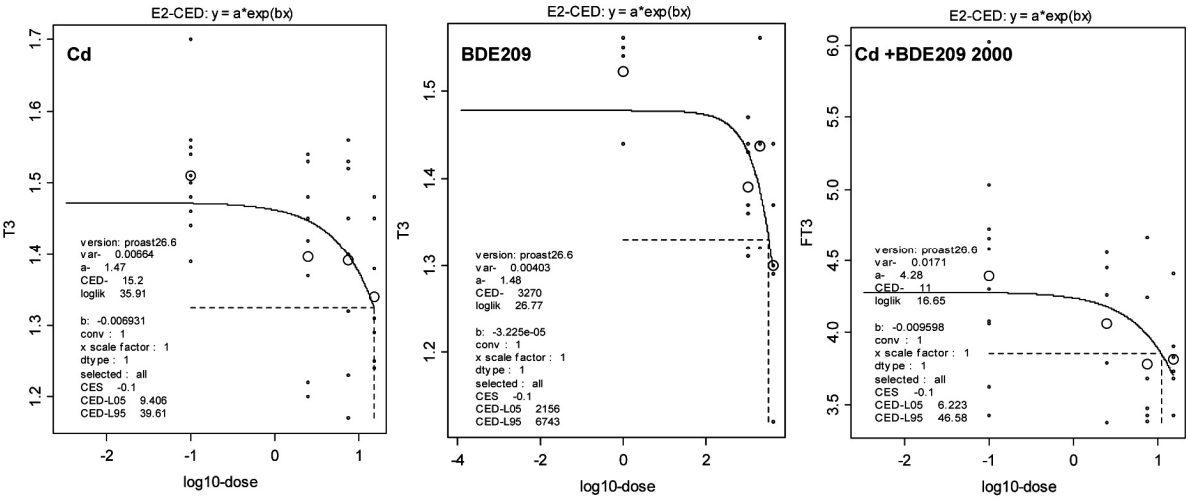

Figure 1. Dose–response curves for the effects on T3 and FT3, analysed against external dose of Cd, BDE209 or mixture of Cd with BDE209.

Parameter *a* is the background (control) level, *b* reflects the efficacy of exposure (slope), *c* maximal response relative to the background level, *d* steepness. Log likelihood is used to assess to what extent the described curve deviates from no effect (*y* = *a*). Critical effect size (CES on the figures), for these endpoints set at 10% (horizontal dotted lines); a corresponding critical effect dose (CED on the figures=Benchmark dose-BMD) is represented by the vertical dotted line; L05 and L95 represent the lower and upper bounds of the 90% confidence interval. Exposure dose (*X*-axis) is on a logarithmic scale.

4. Discussion

Our recently published results have demonstrated that subacute exposure to a mixture of BDE209 and Cd has adverse effects on thyroid hormone homeostasis in adult male rats to a larger extent than BDE209 or Cd alone (22). Application of different doses of BDE209 resulted in statistically significant decrease but in the following hormone levels: FT3, T4 decreased after application of BDE209 in dose of 1000 mg BDE209/kg b.w./day, while FT3,

T4, FT4 after administration of 2000 mg BDE209/kg b.w./day, and T3 after axposure to 4000 mg BDE209/kg b.w./day. Similary, significant decrease in FT4 was obtained after 2.5 mg Cd/kg b.w./day, in FT3, T4 levels after 7.5 mg Cd/kg b.w./day and in FT3 after 15 mg Cd/kg b.w./day. Contribution of mixture was seen when doses of 1000 or 2000 mg BDE209/kg b.w./day were given along with all three doses of Cd inducing decrease in T3, T4 or FT4 levels in comparison with corresponding dose levels of either Cd or BDE209 (22).

Results of different studies have also shown that Cd and BDE209 *per se* may influence thyroid function (30-39). Thyroid dysfunction might be related to structural damage of thyroid follicular cells due to accumulation of Cd in the thyroid gland (40-42), whereas PBDEs have been shown to bind to thyroid receptors (TRs) and perhaps have selective effects on TRs function thus belonging to the group of environmental chemicals that act as thyroid hormone analogues (31, 32, 43-45).

Phenomenon related to combined Cd and BDE209 toxicity was planned to be defined in a dose-related manner by the use of PROAST software (24, 27). Traditionally, when experimental animal data are used for risk assessment of substances, which are not genotoxic and carcinogenic, the NOAEL and/or the Lowest-Observed-Adverse-Effect-Level (LOAEL) for the critical effect of a substance, forms the RP for deriving health-based guidance values, such as an Acceptable Daily Intake (ADI). However, while this approach may utilise qualitative information, it does not use the data available in a quantitative way. In contrast, the BMD approach makes extended use of the dose-response data from studies in experimental animals or from observational epidemiological studies to better characterise and quantify potential risks. After comparing the strengths and weaknesses of the BMD and NOAEL approaches for deriving RP for risk assessment, it has been concluded that the BMD approach is a scientifically more advanced method to the NOAEL approach for deriving a RP, since it makes extended use of available dose-response data and it provides a quantification of the uncertainties in the dose-response data (23). Using the BMD approach also results in a more consistent RP, as a consequence of the specified benchmark response.

In the present study for all treated groups exponential models were fitted, and accordingly BMD and BMDL values were calculated. However, only in the cases where BMD/BMDL ratio was lower than 10, *i.e.* 9.4 mg Cd/kg b.w./day and 2155 mg BDE209/kg b.w./day for the effect on T3; and 6.22 mg Cd/kg b.w./day in mixture with BDE209 2000 mg/kg b.w./day for the effect on FT3, which means that this BMDL could be used as point of departure for further steps in risk analysis (23) (Table I).

5. Conclusion

Application of PROAST software for the effects of Cd and/or BDE209 on thyroid hormone levels confirmed dose-response relationship for both single chemicals and mixture treatment. Some of the resultant BMDL doses may be further assessed and used as a point of depature for establishing health based guidance value.

6. Aknowledgement

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Primena PROAST softvera za ispitivanje uticaja dekabromovanog difeniletra i/ili kadmijuma na homeostazu hormona štitaste žlezde kod pacova

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Kratak sadržaj

Primena statistički dobijene Benchmark doze (BMD) u proceni rizika predstavlja alternativu najčešće korišćenoj „dozi bez štetnog efekta" (NOAEL) zbog veće pouzdanosti u analizi odnosa doze i toksičnog efekta. Cilj ovog rada bio je izračunavanje BMD (10%) primenom PROAST softvera radi kvantitativne procene uticaja Cd i/ili BDE209 na homeostazu hormona štitaste žlezde. Ispitivanje je sprovedeno na mužjacima *Wistar* pacova koji su putem oralne sonde, tokom 28 dana, primali pojedinačne supstance ili njihove kombinacije. Kadmijum je primenjivan u dozama od 2,5, 7,5 i 15 mg Cd/kg t.m./dan, a BDE209 u dozama od 1000, 2000 i 4000 mg BDE209/kg t.m./dan, dok je ostalih devet grupa životinja primalo kombinacije hemikalija (dizajn 3 x 3). Rezultati studije ukazuju da smeša Cd i BDE209 verovatno izaziva intenzivniji poremećaj funkcije štitaste žlezde nego svaka od supstanci pojedinačno. Izračunate Benchmark doze (10%), odnosno odgovarajuće donje granice pouzdanosti (BMDL), uz uslov da je BMD/BMDL < 10, iznose 9,4 mg Cd/kg t.m./dan i 2155 mg BDE209/kg t.m./dan za efekte na T3 hormon, a za efekat na FT3 hormon 6,22 mg Cd/kg t.m/dan u smeši sa BDE209 od 2000 mg/kg.

Ključne reči: doza-odgovor; dekabromovani difeniletar; kadmijum; hormoni štitaste žlezde; smeša.